

# Virology

Volume 134

1984

## EDITORS

W. K. Joklik, EDITOR-IN-CHIEF    G. E. Bruening    P. W. Choppin    R. Haselkorn  
D. W. Kingsbury    A. J. Levine    F. Rapp    M. D. Summers    P. K. Vogt

## ASSOCIATE EDITORS

|                 |                  |                  |                   |                     |                      |
|-----------------|------------------|------------------|-------------------|---------------------|----------------------|
| S. Adhya        | J. M. Dalrymple  | W. S. Hayward    | R. B. Luftig      | H. D. Robertson     | P. G. Spear          |
| C. Baglioni     | J. M. DeMarchi   | K. V. Holmes     | I. G. Miller, Jr. | G. F. Rohrmann      | V. Stollar           |
| A. K. Banerjee  | E. Ehrenfeld     | M. S. Horwitz    | L. K. Miller      | B. Roizman          | B. Sugden            |
| C. Basilio      | F. A. Eiserling  | M. M. Howe       | R. C. Miller, Jr. | J. A. Rose          | M. M. Susskind       |
| K. L. Beemon    | M. K. Estes      | R. Hull          | P. Model          | L. B. Rothman-Denes | R. H. Symons         |
| T. Ben-Porat    | A. J. Faras      | T. Hunter        | T. J. Morris      | J. Sambrook         | P. Tattersall        |
| K. I. Berns     | P. Faulkner      | A. O. Jackson    | B. Moss           | C. E. Samuel        | P. Tegtmeier         |
| D. H. L. Bishop | B. N. Fields     | J. E. Johnson    | S. A. Moyer       | P. A. Schaffer      | D. A. Thorley-Lawson |
| J. M. Bishop    | S. J. Flint      | J. M. Kaper      | F. A. Murphy      | A. Scheid           | D. W. Trent          |
| L. W. Black     | W. R. Folk       | J. D. Keene      | P. E. Neiman      | M. J. Schlesinger   | H. E. Varmus         |
| M. A. Bratt     | R. I. B. Francki | R. M. Krug       | J. R. Nevins      | I. R. Schneider     | I. M. Verma          |
| T. R. Broker    | E. P. Geiduschek | M. Lai           | J. F. Obijeski    | E. M. Scolnick      | E. K. Wagner         |
| J. S. Brugge    | W. Gerhard       | R. A. Lamb       | D. J. O'Callaghan | J. R. Scott         | E. Wimmer            |
| B. J. Carter    | R. F. Gesteland  | R. A. Lazzarini  | P. Palese         | P. B. Sehgal        | O. Witte             |
| J. M. Coffin    | R. M. Goodman    | A. D. Levinson   | E. Paoletti       | P. A. Sharp         | J. Youngner          |
| J. S. Colter    | A. Granoff       | L. Levintow      | C. C. Payne       | J. G. Shaw          | M. Zaitlin           |
| R. J. Courtney  | D. E. Griffin    | D. M. Livingston | H. J. Raskas      | T. Shenk            | N. D. Zinder         |
| S. Dales        | G. Hager         | H. F. Lodish     |                   |                     |                      |



ACADEMIC PRESS, INC.

(Harcourt Brace Jovanovich, Publishers)

San Diego Orlando San Francisco New York London  
Toronto Montreal Sydney Tokyo São Paulo

Copyright © 1984 by Academic Press, Inc.

All Rights Reserved

No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage and retrieval system, without permission in writing from the copyright owner.

The appearance of the code at the bottom of the first page of an article in this journal indicates the copyright owner's consent that copies of the article may be made for personal or internal use, or for the personal or internal use of specific clients. This consent is given on the condition, however, that the copier pay the stated per copy fee through the Copyright Clearance Center, Inc. (21 Congress Street, Salem, Massachusetts 01970), for copying beyond that permitted by Sections 107 or 108 of the U. S. Copyright Law. This consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale. Copy fees for pre-1984 articles are as shown on the article title pages; if no fee code appears on the title page, the copy fee is the same as for current articles.

0042-6822/84 \$3.00

MADE IN THE UNITED STATES OF AMERICA

MICHAEL G. ROSS  
EDUARDO PAEZ AND  
Interference I  
EDUARDO PAEZ AND  
Block Activat  
MARIANO ESTEBAN  
Virus and Rit  
URSULA BANTELS  
Parvovirus Is  
BRIGITTE GEORG-  
Proteins, Hel  
SUSAN B. ALTENB  
Turnip Crinkl  
C. MOUCHES, T. CA  
and Virus-En  
BARBARA NOWAK,  
Physical Map  
a Virion Phos  
ROBERT C. MACDO  
Induced Hemo  
J. K. STRUTHERS,  
Infected Cells  
ZEHAVA GROSSMAN  
40 and Adeno  
HEINZ-JÜRGEN TH  
formation-Sp  
ANITA C. FENTON  
P22 Chromoso  
ANTHONY R. POTE  
P22 .....  
DONALD L. JARVIS  
and Nuclear T  
VICTOR STOLLAR A  
Restricted in  
GRETCHEN B. CAU  
tural Proteins  
M. ETZERODT, T. M  
Sequence of t  
DAVID D. AUPERI  
and Intergen  
RICKY R. HIRSCHH  
of Deletion M  
SHORT COMMUNICA  
A. K. GITELMAN  
in the Ar  
ÉVA ÁDÁM AND  
in the Ad  
A. KIUCHI AND  
Protein w  
STEVEN G. IRV  
AND MICHAEL  
by Pulmo  
C. W. WARD,  
Segment

# Characterization of the DNA of a Defective Human Parvovirus Isolated from a Genital Site

URSULA BANTEL-SCHAAL,\* AND HARALD ZUR HAUSEN\*†<sup>1</sup>

\*Institut für Virologie, Zentrum für Hygiene, Universität Freiburg, Hermann-Herder-Straße 11,  
D-7800 Freiburg, and †Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280,  
D-6900 Heidelberg, West Germany

Received August 25, 1983; accepted January 4, 1984

An apparently helper-dependent parvovirus was isolated from a penile flat condylomatous lesion by inoculating the material into a culture of human fibroblasts, and by coinfecting these cells with adenovirus type 12. Upon addition of an adenovirus helper, the virus could be readily propagated in human KB cells. Analysis of its DNA revealed that it shares structural similarities with the DNA of the four defective parvoviruses (adeno-associated viruses, AAV) described so far, but that the cleavage pattern obtained after digesting the DNA with restriction enzymes is different from that of AAV 1-AAV 4. Nucleic acid hybridization data further support the assumption that the isolated defective parvovirus is not identical with the other four serotypes. When conditions of high stringency were used, only weak cross-reactivity was seen with the DNAs of AAV 1-AAV 4, whereas the latter showed strong cross-hybridization with each other. The organization of the DNA of the newly isolated parvovirus was investigated by mapping the cleavage sites of several restriction enzymes. It is proposed to designate the new isolate as AAV 5.

## INTRODUCTION

In the course of experiments to study the possible influence of adenovirus infection on replication of papilloma viruses in tissue culture cells, an apparently helper-dependent small virus was isolated from a penile flat condylomatous lesion. From its size and the observed helper dependence on adenoviruses for its replication, it was concluded to be an adeno-associated virus (AAV).

Adeno-associated viruses are members of the parvoviridae family and are defective in that they require coinfection with adenovirus or herpesvirus for productive infection (Atchison *et al.*, 1965; Hoggan *et al.*, 1966; Buller *et al.*, 1981). The genome of AAV is a linear, single-stranded DNA with the complementary strands separately encapsidated into individual virions with equal frequency. Upon proper conditions, these strands anneal to form linear duplex molecules (Mayor *et al.*, 1969; Rose

*et al.*, 1969; Berns and Rose, 1970; Berns and Adler, 1972).

Adeno-associated viruses are interesting in several respects. Since the DNA is small (about 4.7 to 4.8 kb in size; Muster *et al.*, 1980), it provides a good model system to study genome organization and gene expression in eucaryotes. Furthermore, it shares characteristics with insertion elements in that its genome has both an inverted and a direct terminal repetition (Gerry *et al.*, 1973; Kocot *et al.*, 1973; Fife *et al.*, 1977; Lusby *et al.*, 1980; Lusby *et al.*, 1981). Actually, when no helper virus is present, AAV DNA can persist in cells of at least some continuous cell lines from which it can be rescued upon addition of helper virus (Cheung *et al.*, 1980).

In addition to these features, adeno-associated viruses have been reported to interfere with virally induced oncogenicity and cell transformation (Kirschstein *et al.*, 1968; Casto and Goodheart, 1972; Mayor *et al.*, 1973; Blacklow *et al.*, 1978; de la Maza and Carter, 1981; Ostrove *et al.*, 1981). The same holds true for the nondefective par-

voviruses virally induced formation of ring tumor (Ledinko, 1982; Toola

In this re particles is belong to t ruses with dence and t of the DN from the fo 4) that have so far (Atcl 1966; Park: size of its D The new is as AAV 5.

MA

Cells. Hu cells were containing streptomyc mented in cells were subcultured ditions for ham *et al.*, monkey ki tially the s FCS added for GMK c

Viruses (helper: ad (helper: ad adenovirus 15) were ob Culture Co propagation: 1 (helper: : AAV 4 (he 2 H (helpe (helper: ad (helper: ad also used f type 2 and was assaye nucleic aci The AAV 30 min to i

<sup>1</sup> To whom reprint requests should be addressed.

voviruses which inhibit chemically and virally induced oncogenesis and cell transformation as well as spontaneously occurring tumors (Toolan, 1967; Toolan and Ledinko, 1968; Mousset and Rommelaere, 1982; Toolan *et al.*, 1982).

In this report we describe that the small particles isolated from the genital lesion belong to the group of defective parvoviruses with regard to their helper dependence and the DNA structure. Comparison of the DNA shows that the virus differs from the four AAV serotypes (AAV 1-AAV 4) that have been found in man and monkey so far (Atchison *et al.*, 1965; Hoggan *et al.*, 1966; Parks *et al.*, 1967), not only in the size of its DNA but also in DNA homology. The new isolate is tentatively designated as AAV 5.

#### MATERIAL AND METHODS

**Cells.** Human foreskin fibroblasts and KB cells were maintained in Eagle's MEM containing 5% fetal calf serum (FCS), streptomycin, and penicillin, and supplemented in addition with glutamine. The cells were grown in monolayers and were subcultured once or twice a week. The conditions for cultivating of 293-31 cells (Graham *et al.*, 1977) and the African green monkey kidney cells (GMK) were essentially the same except for the amounts of FCS added (10% for 293-31 cells and 3% for GMK cells, respectively).

**Viruses and virus infections.** AAV 1 (helper: adenovirus type 7a), AAV 2 H (helper: adenovirus type 2), AAV 3 (helper: adenovirus type 2) and AAV 4 (helper: SV 15) were obtained from the American Type Culture Collection (ATCC). Cells used for propagation of virus were 293-31 for AAV 1 (helper: adenovirus type 7a); GMK for AAV 4 (helper: SV 15); and KB for AAV 2 H (helper: adenovirus type 2), AAV 3 (helper: adenovirus type 2), and AAV 5 (helper: adenovirus type 12). KB cells were also used for propagation of adenoviruses type 2 and 12. The adenovirus type 12 stock was assayed to be free of AAV 5 by using nucleic acid hybridization techniques.

The AAV inoculum was kept at 56° for 30 min to inactivate the helper virus, and

new helper virus was added for coinfections. Adsorption of virus suspension was for 1 hr and then fresh medium was added. The cells were incubated at 37° for approximately 3 days. At this time, most of the cells showed cytopathogenic changes. Cells and culture fluid were subjected to three rounds of freezing and thawing and then used for virus purification.

**Virus purification.** Cell debris was removed by low-speed centrifugation and ammonium sulfate up to 40% saturation was added to the supernatant. The resulting precipitate was sedimented by centrifugation, redissolved in phosphate-buffered saline (PBS), and dialyzed against this buffer. The material obtained after dialysis was kept at 56° for 30 min, and was then treated with DNase (Boehringer, Mannheim, 2000 U/mg) and RNase (Merck, Darmstadt, 25 U/mg) at 37° for 1 hr (2 µg/ml of each enzyme). The resulting suspension was overlaid a CsCl cushion (14 ml CsCl,  $\rho_{\text{CsCl}} = 1.3 \text{ g/cm}^3$ ) and sedimented by centrifugation in a Beckman SW 27 rotor for 16 hr at 16,000 rpm. The pellet was resuspended and the suspension was brought to a density of  $\rho_{\text{CsCl}} = 1.22 \text{ g/cm}^3$ , and then centrifuged in the Beckman SW 41 rotor for 16 hr at 16,000 rpm.

**Control of infectivity.** During purification, aliquots of AAV 5 suspension were taken at individual steps of the procedure (supernatant after removing of cell debris, suspension after dialysis, and resuspended pellet after the SW 27 run and after the SW 41 run) and were assayed for infectivity. The aliquots were heated at 56° for 30 min and then mixed with adenovirus type 12 stock virus as helper. KB cells were infected as described above and particle production was monitored by electron microscopy. Additionally, in some experiments infected cells were incubated for about 20 hr, then harvested and analyzed for the expression of AAV antigens by immunofluorescence (Henle and Henle, 1966). The monoclonal antibody directed against AAV 5 capsid proteins is described elsewhere (Georg-Fries *et al.*, 1984).

**Extraction and purification of viral DNA.** Pelleted virus was resuspended and the DNA was extracted by treating the sus-

pension with 0.1 N NaOH, 0.15% Sarkosyl, and 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) for 1 hr at room temperature (Berns *et al.*, 1975). The solution was neutralized with 0.2 N HCl and then treated with proteolytic enzymes (trypsin, 10 µg/ml, 1 hr, 27°, 33 U/mg; proteinase K, 10 µg/ml, 1 hr, 37°, 20 U/mg; both enzymes were from Boehringer, Mannheim). Desalting, removal of proteins, and concentration of DNA were done by chromatographing the solution on a small Sephadex G 50/malachite green gel column (Bünnemann and Müller, 1978; Koller *et al.*, 1978). Care has to be taken to sufficiently dilute the solution before loading it onto the malachite green gel since higher salt concentrations will hinder the binding of DNA to the affinity material. The buffer used for equilibrating and washing of the column and for diluting the DNA was 10 mM Tris, 1 mM EDTA, pH 6.5. Elution was done with 2 M sodium perchlorate, 10 mM Tris, pH 7.5.

**Gel electrophoresis.** DNA was electrophoresed on agarose (SeaKem) slab gels (Sharp *et al.*, 1973). Separation of fragments of restriction enzyme digest was additionally performed on a 3.5 to 13% polyacrylamide gradient gel (acrylamide:bisacrylamide, 29:1) (Jeppesen, 1980). For preparative purposes, 1% low-melting-temperature agarose (LMT agarose) (Sea-Plaque) gels were used. The LMT agarose was sterilized and the gels were poured in the cold room. Gels were run in a buffer of pH 7.8 containing 40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA. PM 2 DNA, *Hind*III-cleaved, and  $\phi$ X174 RF DNA, *Hae*III-cleaved, were used as size markers. For staining, the gels were soaked in an ethidium bromide solution (0.5 µg/ml) for 30 min (Sharp *et al.*, 1973).

**Preparation of AAV duplex DNA.** Annealing was done by heating the DNA eluted from the malachite green gel/Sephadex G 50 column at 65° for 15 min and then slowly cooling the solution to room temperature. The DNA was fractionated by electrophoresis on 1% LMT agarose gels. From these gels DNA was recovered according to the method described by Weislander (1979). If necessary, the DNA was

concentrated prior to electrophoresis by sedimenting it in an air-driven ultracentrifuge (Airfuge, Beckman) at 76,000g for 75 min. To reduce convection, sedimentation was performed in a solution containing small amounts of CsCl ( $n_D^{20} = 1.3362$ ). For nick-translation, double-stranded DNA was not extracted from the LMT agarose. Instead the method of Parker and Seed (1980) for sequential digest was applied. The gel slice containing the DNA was cut out and melted by heating it to 65° (buffer, salt, and water added). It then was further diluted by adding the dNTPs at 37° before starting the enzymatic reaction at 17°.

**Nick-translation.** Nick-translation was achieved by the procedure of Kelly *et al.* (1970) and Rigby *et al.* (1977). Radiolabeled [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\alpha$ -<sup>32</sup>P]dTTP were purchased from Amersham Buchler, Braunschweig.

**Transfer of DNA onto nitrocellulose filters.** The DNA was transferred to nitrocellulose filters (Schleicher and Schüll, BA 85) as described by Southern (1975). If identical nitrocellulose filters were needed for hybridization with two different probes, bidirectional transfer was performed according to Smith and Summers (1980).

**Filter hybridization.** Filter hybridization was carried out at 42° according to the technique of Denhardt (1966). The hybridization solution contained 50% formamide, 5 × SSC (0.75 M NaCl, 0.075 M sodium citrate), 20 mM sodium phosphate, pH 6.5, 5 × Denhardt's solution (Denhardt, 1966), and 100 µg/ml t-RNA in addition to the <sup>32</sup>P-radiolabeled DNA. After hybridization, the filters were washed at 70° in a solution containing 1 × SSC and 0.1% sodium dodecyl sulfate for 90 min with three subsequent changes of buffer, and then autoradiographed by using a Kodak X Omat AR film with intensifying screen at -70°.

**Digestion with endodeoxyribonucleases (restriction enzymes).** The enzymes used for digestion of AAV duplex DNA were *Bam*HI, *Eco*RI, *Hinc*II, *Hind*III, *Hpa*I, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I, and *Xho*I. With the exception of *Hpa*I and *Kpn*I (New England Biolabs), all others were purchased from Bethesda Research Laboratories. Enzymatic digestions were carried

out at 37° respectively to the reco  
*et al.* (1982  
zymes we  
buffers pri  
solution, se  
tween 0.8 a  
carried out  
were activ  
tively, the  
lower ionic  
then the r  
second enz  
ate amoun

Propagatio

Superfici  
dylomatou



FIG. 1.  
cells (ad  
sediment  
SW 41 ro  
(b) parti

oresis by ultracentrifugation at 100,000g for 2 hours (containing 362). For agarose and Seed applied. was cut (buffer, further 7° before at 17°. tion was ally *et al.* liolabeled ere pur- r, Braun-

*Tulose fil-*  
nitrocel-  
schüll, BA  
(1975). If  
re needed  
nt probes,  
rmed ac-  
(1980).  
ridization  
ng to the  
ie hybrid-  
rmamide,  
M sodium  
te, pH 6.5,  
rdt, 1966),  
on to the  
ridization,  
a solution  
odium do-  
three sub-  
then au-  
k X Omat  
n at -70°.  
*nucleases*  
es used for  
NA were  
(II, *Hpa*I,  
and *Xho*I.  
*Kpn*I (New  
were pur-  
h Labora-  
re carried

out at 37° in final volumes of 10 and 14  $\mu$ l, respectively. Buffers were used according to the recommendations made by Maniatis *et al.* (1982). For mapping experiments, enzymes were diluted in the appropriate buffers prior to adding them to the DNA solution, so that their activities ranged between 0.8 and 1.5 U. Double digestions were carried out simultaneously if both enzymes were active in the same buffer. Alternatively, the enzyme reactive in a buffer of lower ionic strength was used initially, and then the reaction was continued with the second enzyme after adding the appropriate amount of salt.

## RESULTS

### *Propagation of Virus*

Superficial scrapings from a flat condylomatous lesion of the penis were sus-

pended in Eagle's MEM containing 5% FCS and added to a culture of human foreskin fibroblasts (line  $\alpha$ 1). The cells were coinfecting with adenovirus type 12 at an input multiplicity of approximately 10 PFU/cell. The cytopathogenic changes were typical for adenovirus but showed a remarkable delay, with alterations first noted 5 days after infection. Only few adenovirus particles were observed when the supernatant of the pelleted culture fluid was examined by electron microscopy. In addition, however, high concentrations of small particles about 20 nm in diameter were noted. In control cultures infected with adenovirus type 12, no additional virus was discovered. Subcultivation of the virus suspension in human KB cells resulted in a further increase of the small particles within 3 days after infection. No evidence was obtained

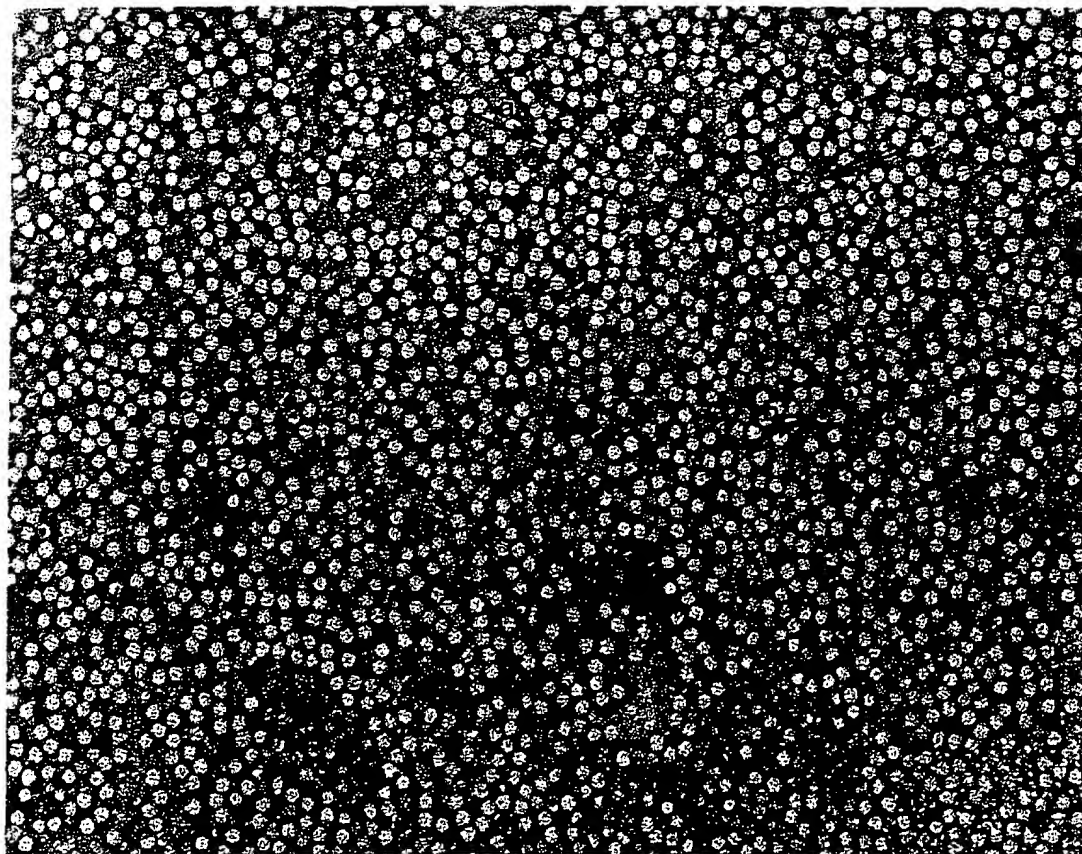


FIG. 1. Electron micrograph of AAV 5 particles. AAV 5 was isolated from infected KB monolayer cells (adenovirus type 12 used as helper) and concentrated by ammonium sulfate precipitation and sedimentation in the ultracentrifuge. The final centrifugation step through low-density CsCl in the SW 41 rotor yields a pellet that contains AAV particles of all density classes ("full" (a) and "empty" (b) particles), and the small capsomers (c) of the destroyed helper virus.

for helper-independent replication of the small virus after prior inactivation of the adenovirus by keeping the virus suspension at 60° for 30 min.

The size of the particles and the apparent dependence on a helper virus for its replication suggested that the virus belongs into the group of defective parvoviruses (adeno-associated viruses, AAV).

#### Virus Purification

Since adeno-associated viruses are known to consist of a population of various density classes (Hoggan *et al.*, 1966; Hoggan, 1971), conditions for the purification procedure intended to avoid the exclusion of any of these classes. Therefore, the virus suspension was sedimented through a CsCl cushion and finally was pelleted through low-density CsCl in a SW 41 rotor. The pellet of the final centrifugation step thus should contain all kinds of virus particles and, upon DNA extraction, should directly lead to the variant AAV DNAs (Laughlin *et al.*, 1979; de la Maza and Carter, 1980) in addition to DNA of unit length (Fig. 2).

As seen in Fig. 1, virus particles of the adenovirus helper are destroyed during the course of purification, probably due to high salt concentrations and heating. Capsomer units are still present. After application of DNase, the amounts of contaminating adenovirus DNA are only small (Figs. 2, 3A).

Infectivity of AAV 5 remains intact throughout the purification procedure, as judged by taking the material obtained after different steps for infection tests and then analyzing it by electron microscopy. Similarly, when AAV antigen expression was studied by indirect immunofluorescence the results led to the same conclusion. In either case, no significant decrease of infectious particles was observed after the individual steps of purification (data not shown).

#### Viral DNA

The majority of DNA eluted from the malachite green gel/Sephadex G 50 column consisted of single strands, as judged from agarose gels (data not shown). Upon annealing, a number of different physical forms were obtained in addition to unit

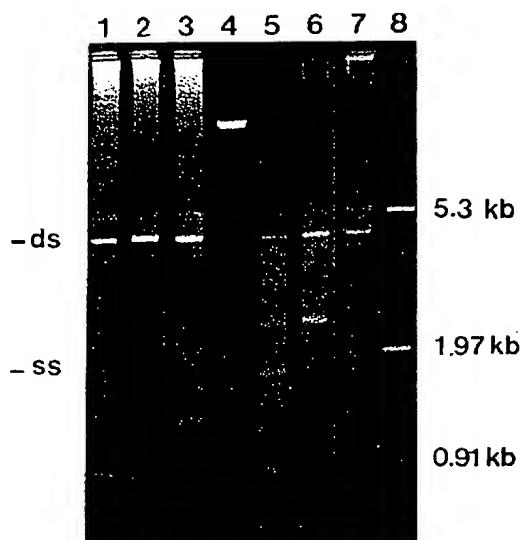


FIG. 2. Fractionation of AAV DNAs in agarose gels. The DNAs of different types of AAV were extracted from the virus particles as described. After heating to either 37° (lanes 5, 6, 7) or 65° (lanes 1, 2, 3) with subsequent slow cooling to room temperature, they were electrophoretically separated in a 1% agarose gel. Lanes 1 and 5, AAV 5 DNA; lanes 2 and 6, AAV 3 DNA; lanes 3 and 7, AAV 1 DNA; lane 4, adenovirus type 12 DNA; lane 8, PM 2 DNA, *Hind*III-cleaved as size marker. ds, unit-length duplex monomers; ss, single-strand DNA; kb, 10<sup>3</sup> bp.

length, linear duplex DNA. These forms were known to contain unit length, duplex circles and linear duplex dimers besides a number of other less well-defined structures (Gerry *et al.*, 1973; Koczot *et al.*, 1973). Heating at 65° for 15 min followed by slow cooling to room temperature converted the single-stranded DNA into the duplex form but, additionally, lead to formation of structures that did not enter a 1% agarose gel (Fig. 2). This phenomenon was also seen when the DNA was concentrated by precipitation or sedimentation in an air-driven ultracentrifuge, and was probably due to oligomeric network-like structures of AAV DNA originating in properties contributed by the terminal structures (Gerry *et al.*, 1973; Koczot *et al.*, 1973).

During the purification of virus no selection was made with regard to particle density. Thus, after DNA extraction molecules of less than genome length were present originating from defective interfering particles (de la Maza and Carter, 1980). As can be seen from Fig. 2, this vari-

FIG. 3  
AAV D  
one nitr  
labeled  
DNA. P  
then wa  
as size  
AAV 3

ant DNA  
bands in  
mained co  
agation (c  
distinct ty  
preferenti  
in the vir

Comparis  
AAV

Compar  
DNAs of  
4 in agar  
DNA was  
4.6 kb as  
distance, I  
pattern re  
defective  
and Carte  
serotype (c  
DNA con  
smaller th  
AAV 3 D

5.3 kb  
1.97 kb  
0.91 kb

agarose gels.  
e extracted  
ter heating  
1, 2, 3) with  
ature, they  
1% agarose  
and 6, AAV  
adenovirus  
I-cleaved as  
monomers; ss,

se forms  
ch, duplex  
s besides  
ned struc-  
al, 1973).  
ed by slow  
verted the  
plex forma-  
tion of  
% agarose  
s also seen  
ed by pre-  
air-driven  
oly due to  
es of AAV  
ontributed  
erry *et al.*,

rus no se-  
to particle  
ction mol-  
ngth were  
tive inter-  
nd Carter,  
; this vari-

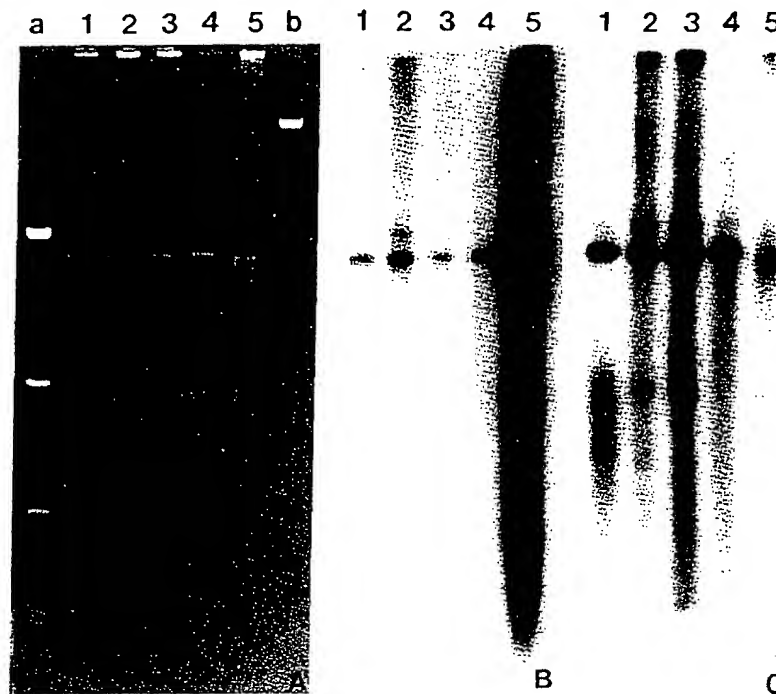


FIG. 3. Comparison of the DNAs of the different AAV serotypes. (A) Fractionation of noncleaved AAV DNAs in a 1% agarose gel stained with ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ). (B) Hybridization of one nitrocellulose filter obtained from the same gel after bidirectional Southern transfer with  $^{32}\text{P}$ -labeled AAV 5 duplex DNA. (C) Hybridization of the identical filter with  $^{32}\text{P}$ -labeled AAV 3 duplex DNA. Prior to radiolabeling, the DNA was fractionated on LMT agarose gels and the duplex DNA then was nick-translated directly from the gel as described. Lane a, PM 2 DNA, *Hind*III-cleaved as size marker; lane b, adenovirus type 12 DNA; lane 1, AAV 1 DNA; lane 2, AAV 2 DNA; lane 3, AAV 3 DNA; lane 4, AAV 4 DNA; lane 5, AAV 5 DNA.

ant DNA exhibited a pattern of distinct bands in an agarose gel. The pattern remained constant for repeated virus propagation (data not shown), suggesting that distinct types of variant DNA were either preferentially made or/and encapsidated in the virus particles.

#### *Comparison of the DNAs of the Different AAV Serotypes*

Comparison of the AAV 5 DNA with the DNAs of the four serotypes AAV 1-AAV 4 in agarose gels revealed that the AAV 5 DNA was of a slightly smaller size (4.5 to 4.6 kb as calculated from the migration distance, Fig. 3A). As expected, the banding pattern representing the variant DNAs of defective interfering particles (de la Maza and Cartér, 1980) was different for each serotype (Fig. 2). Preparations of AAV 5 DNA contained far more DNA of a size smaller than unit length than seen, e.g., in AAV 3 DNA preparations (Fig. 2). Upon

reannealing, this led to a heterogenous population of molecules in addition to the predominant species that exhibited the distinct banding pattern. In hybridization experiments with the homologous  $^{32}\text{P}$ -labeled DNA, this resulted in labeling of the complete lane on the filter. As expected from the smaller quantity of variant DNA, this phenomenon was less pronounced with AAV 3 (Figs. 3B, lane 5, and C, lane 3).

In blot-hybridization experiments with  $^{32}\text{P}$ -radiolabeled AAV 5 duplex DNA, only a weak cross-hybridization with the other four serotypes was seen under stringent conditions. This cross-reactivity was essentially confined to the bands representing complete genomes (Fig. 3B). Additionally, a very weak signal appeared at the position where the variant DNA of the largest size migrated when long times of exposure were used. A similar picture emerged with regard to AAV 5 DNA when hybridization of an identical filter was

performed with  $^{32}\text{P}$ -labeled AAV 3 duplex DNA (Fig. 3C). The cross-hybridization occurred at the positions representing the complete AAV 5 genome and the variant AAV 5 DNA of the largest size. Obviously, the serotypes AAV 1-AAV 4 shares more homologies with each other than they do with AAV 5, since all of them rather strongly cross-hybridized with AAV 3 duplex DNA. In addition, the reaction was extended to far more of the variant DNAs than in the case of AAV 5 (Fig. 3C). These findings were supported by the results shown in Fig. 5B. While  $^{32}\text{P}$ -labeled AAV 3 duplex DNA showed cross-hybridization with all fragments obtained from restriction-enzyme digested AAV 2 DNA, it did not react with the terminal fragments of AAV 5 DNA (*Xho*I B-fragment, *Bam*HI C-fragment, cf. Fig. 7).

Digesting the isolated double-stranded AAV DNAs with restriction enzymes yielded different restriction patterns for all five serotypes, as seen in Figs. 4 and 5. The cleavage sites observed with the endonucleases *Bam*HI and *Xho*I for the new isolate clearly differed from those seen for

AAV 1, AAV 2, AAV 3, and AAV 4 (Figs. 4, 5). In addition, no identical cleavage pattern was obtained when the DNAs of AAV 1, AAV 3, AAV 4, and AAV 5 were digested by the enzyme *Hinc*II (Fig. 4).

#### Restriction Enzyme Patterns and Size of Fragments for AAV 5 DNA

Isolated AAV 5 duplex DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hinc*II, *Hind*III, *Hpa*I, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I, and *Xho*I. Within the resolution of a 2% agarose gel, no cleavage site has been found with the enzymes *Hind*III, *Hpa*I, *Kpn*I, *Pst*I, and *Xba*I. For the other restriction enzymes used, the number of fragments and their sizes, as estimated from 2% agarose gels (data not shown) and 3.5 to 13% polyacrylamide gels (Fig. 6), are listed in Table 1. With the technique used (Southern transfer and filter hybridization) the smallest fragment seen from agarose gels in X-ray films was about 220 bp in size (*Sma*I C-fragment), and in some cases the *Xho*I C-fragment with a size of about 140 bp could

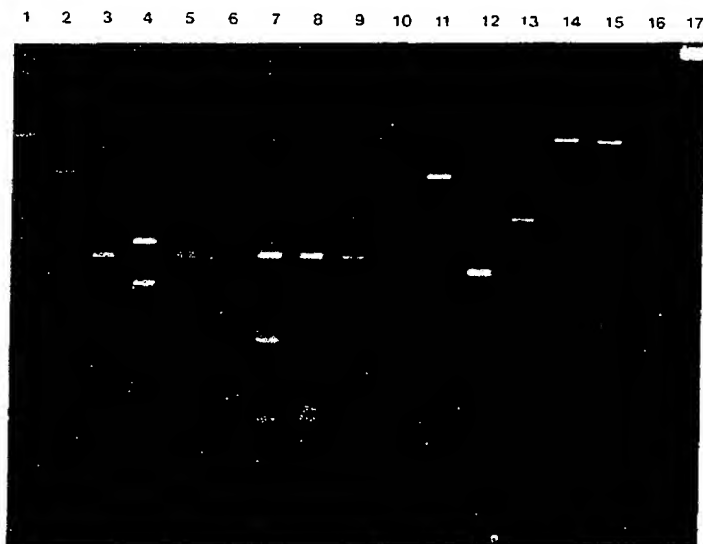


FIG. 4. Digestion of AAV DNAs with restriction enzymes. The duplex DNA of AAV 1, AAV 3, AAV 4, and AAV 5 was isolated from agarose gels and cleaved with the restriction enzymes *Bam*HI, *Hinc*II, and *Xho*I at 37° in a final volume of 14  $\mu\text{l}$ . Lanes: 1. AAV 5, uncleaved; 2. AAV 5, *Xho*I; 3. AAV 4, *Xho*I; 4. AAV 3, *Xho*I; 5. AAV 1, *Xho*I; 6. AAV 5, *Hinc*II; 7. AAV 4, *Hinc*II; 8. AAV 3, *Hinc*II; 9. AAV 1, *Hinc*II; 10. AAV 5, *Bam*HI; 11. AAV 4, *Bam*HI; 12. AAV 3, *Bam*HI; 13. AAV 1, *Bam*HI; 14. AAV 4, uncleaved; 15. AAV 3, uncleaved; 16. AAV 1, uncleaved; 17. adenovirus type 12 DNA, uncleaved.

1 2 3

A

FIG. 5. Dig with restric 2 and AAV striction en: Fig. 4. The fi to nitrocellu with  $^{32}\text{P}$ -lab (B). Since t DNA and A the AAV 5 l Thus the cr and AAV 5 shown in (A) uncleaved; 3 AAV 2, *Xho*.

be seen af The lower tectable u acrylamid lane 15) a ment four was indee

Mapping Sites

Mappin sites was *Bam*HI, i

(Figs.  
ge pat-  
f AAV  
igested

Size of

as di-  
cleases

*HpaI*,

*XhoI*.

ose gel,

ith the

tl, and

zymes

d their

se gels

yacryl-

able 1.

trans-

mallest

X-ray

mal C-

*XhoI* C-

p could

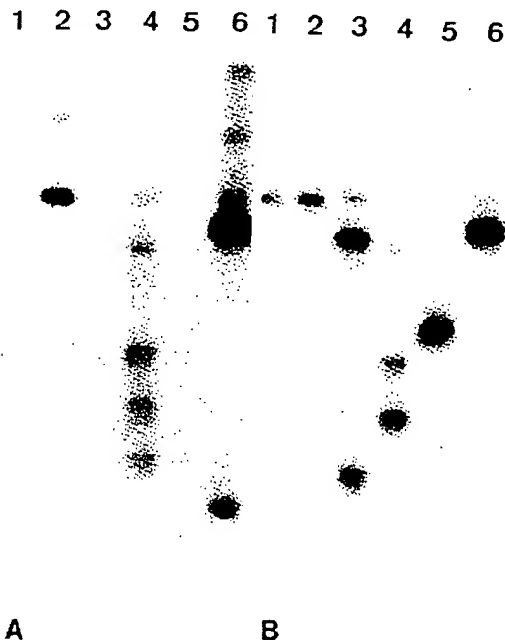


FIG. 5. Digestion of AAV 2 DNA and AAV 5 DNA with restriction enzymes. The duplex DNA of AAV 2 and AAV 5 was isolated and cleaved with the restriction enzymes *Bam*HI and *Xho*I as described in Fig. 4. The fragments were bidirectionally transferred to nitrocellulose filters and the filters were hybridized with  $^{32}$ P-labeled AAV 5 DNA (A) and AAV 3 DNA (B). Since the cross-hybridization between AAV 3 DNA and AAV 5 DNA is rather weak (cf. Fig. 3C), the AAV 5 DNA was used in higher concentrations. Thus the cross-hybridization between AAV 2 DNA and AAV 5 DNA is not seen at the time of exposure shown in (A). Lanes: 1. AAV 2, uncleaved; 2. AAV 5, uncleaved; 3. AAV 2, *Bam*HI; 4. AAV 5, *Bam*HI; 5. AAV 2, *Xho*I; 6. AAV 5, *Xho*I.

be seen after prolonged times of exposure. The lower limit for fragment size still detectable unambiguously from blotted polyacrylamide gels was about 100 bp (Fig. 6, lane 15) and with this, the smallest fragment found with the single enzyme digest was indeed the *Xho*I C-fragment.

#### Mapping of Restriction Enzyme Cleavage Sites on AAV 5 Duplex DNA

Mapping of restriction enzyme cleavage sites was accomplished for the enzymes *Bam*HI, *Eco*RI, *Hinc*II, *Sal*I, *Sma*I, and

*Xho*I. Double and partial digests were performed and the resulting fragments were separated on agarose gels (data not shown) and polyacrylamide gradient gels (Fig. 6), from which they were transferred to nitrocellulose filters and visualized by hybridization with  $^{32}$ P-labeled AAV 5 DNA.

The map derived from these data is shown in Fig. 7. As the minus strand of AAV 5 DNA has not yet been determined, no orientation of the map according to the

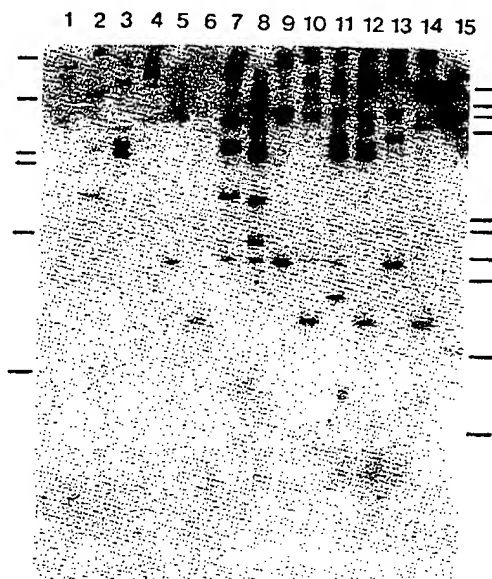


FIG. 6. Cleavage of AAV 5 double-stranded DNA with different restriction enzymes. AAV 5 duplex DNA was isolated and cleaved with different restriction enzymes at 37° for 1 hr in a final volume of 10  $\mu$ l. Enzymatic activities in the test were between 0.8 and 1.5 U. Buffers were as mentioned in the text. The fragments were separated in a 3.5 to 13% polyacrylamide gel with a 3% stacking gel, and then transferred to nitrocellulose filters as described and visualized by hybridization with  $^{32}$ P-radiolabeled AAV 5 DNA. Size markers were PM 2 DNA, *Hind*III-cleaved (left track) and  $\phi$ X174 RF DNA, *Hae*III-cleaved (right track) (cf. Table 1), with a resolution of 1.97 to 0.1 kb (PM 2 marker) and 1.35 to 0.072 kb ( $\phi$ X174 marker). The C-fragment of the *Sma*I digest still seen in lane 11 (double-digest *Sma*I and *Hinc*II) is due to incomplete digest since it usually was not seen in polyacrylamide gels of this double digest. Lanes: 1. *Bam*HI; 2. *Eco*RI; 3. *Hinc*II; 4. *Sal*I; 5. *Sma*I; 6. *Xho*I; 7. *Bam*HI and *Eco*RI; 8. *Bam*HI and *Hinc*II; 9. *Bam*HI and *Sma*I; 10. *Bam*HI and *Xho*I; 11. *Hinc*II and *Sma*I; 12. *Hinc*II and *Xho*I; 13. *Sal*I and *Sma*I; 14. *Sal*I and *Xho*I; 15. *Sma*I and *Xho*I.

AV 3,  
nHI,  
ol; 3.  
ncII;  
mHI;  
DNA,

TABLE 1

SIZE OF AAV 5 DNA RESTRICTION FRAGMENTS AS ESTIMATED FROM 2% AGAROSE GELS AND 3.5 TO 13% POLYACRYLAMIDE GELS

| Enzyme         | Fragment size (base pairs $\times 10^3$ ) |      |      |      |      |      | Total |
|----------------|---|------|------|------|------|------|-------|
|                | A   | B    | C    | D    | E    | F    |       |
| <i>Bam</i> HI  | 1.85                                      | 1.40 | 1.03 | 0.23 | —    | —    | 4.51  |
| <i>Eco</i> RI  | 2.65                                      | 1.10 | 0.47 | 0.34 | —    | —    | 4.56  |
| <i>Hinc</i> II | 1.32                                      | 0.95 | 0.65 | 0.56 | 0.50 | 0.50 | 4.48  |
| <i>Sal</i> I   | 2.95                                      | 1.60 | —    | —    | —    | —    | 4.55  |
| <i>Sma</i> I   | 3.55                                      | 0.78 | 0.22 | —    | —    | —    | 4.55  |
| <i>Xho</i> I   | 3.65                                      | 0.76 | 0.14 | —    | —    | —    | 4.55  |

Note. PM 2 DNA, *Hind*III-cleaved and the DNA of  $\phi$ X174 RF, *Hae*III-cleaved, were used as size markers. Fragment size of marker DNA (base pairs  $\times 10^3$ ): PM 2 DNA, *Hind*III-cleaved; 5.3, 1.97, 0.91, 0.44, 0.42, 0.26, and 0.1.  $\phi$ X174 RF DNA, *Hae*III-cleaved; 1.35, 1.08, 0.87, 0.6, 0.31, 0.271/0.281, 0.234, 0.194, 0.118, and 0.072.

convention by Ward and Tattersall (1978) was made. Since the smallest fragment that could be identified with certainty by

the technique used was about 100 bp in size, the small fragments resulting from the double digests *Hinc*II/*Sma*I (about 50 bp) and *Hinc*II/*Xho*I (about 50 bp), and one fragment expected from the *Sma*I/*Xho*I digest (about 20 bp) were not seen.

## DISCUSSION

The experiments presented in this report describe a new type of a helper-dependent parvovirus. With regard to its helper dependence and its DNA structure, this virus clearly belongs into the group of adeno-associated viruses and is tentatively labeled as AAV 5.

Adeno-associated viruses are known to give rise to several visible bands when centrifuged to equilibrium in CsCl buoyant density gradients (Hoggan *et al.*, 1966; Hoggan, 1971). In addition to these bands, the spectrum of virus particles is also extended to the interbanding regions (de la Maza and Carter, 1980). We therefore chose a purification procedure that avoids exclusion of any density group. Thus, upon DNA

extraction DNA molec the variant 1980) leads in agarose directly ext from the g ited by the for each in constant up This high mechanism: site-specific from prefe species of 1

Compari isolated def the DNAs c by blot-hyl only a weal is confined complete g to the vari: the other bridization AAV serot to smaller These findi lated parve AAV 1-AA AAV 5 is m adeno-asso among each show incre gions as th genome ter 1980), the h 5 and AAV within the tion is sup diolabeled hybridizati ment of the C-fragmen zyme *Bam*.

The conc duplex DN usually is r fore a sens termine tl gesting the striction er transfer tl

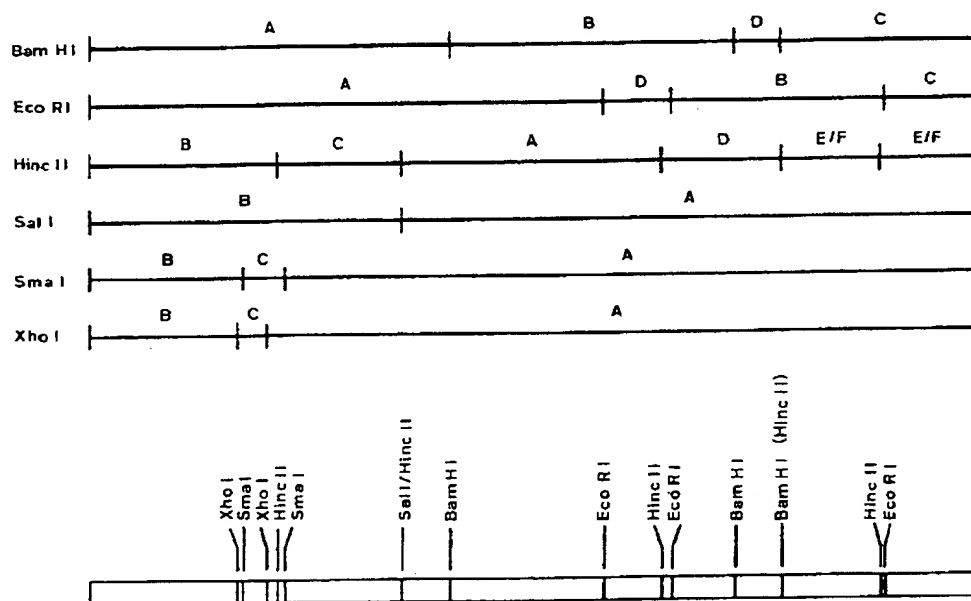


FIG. 7. Map of restriction endonuclease cleavage sites in the DNA of AAV 5. The cleavage sites of the restriction endonucleases *Bam*HI, *Eco*RI, *Hinc*II, *Sal*I, *Sma*I, and *Xho*I were determined. Fragments are designated by alphabetic letters. Their respective size is listed in Table 1. The fragments E and F of the *Hinc*II cleavage comigrate in the agarose gel and are also not resolved in the polyacrylamide gel. Thus, they were not distinguished. The cleavage sites for the enzyme *Bam*HI (D/C) and for the enzyme *Hinc*II (D/E(F)) are not resolved since they are very close to each other.

extraction the whole population of AAV DNA molecules was obtained (Fig. 2). Since the variant DNA (de la Maza and Carter, 1980) leads to a distinct banding pattern in agarose gels (Fig. 2), it is possible to directly extract specific forms of AAV DNA from the gels. The banding pattern exhibited by the variant DNAs is characteristic for each individual serotype and remains constant upon repeated virus propagation. This might originate either from the mechanism of DNA synthesis or from a site-specific cleaving of the DNA, or else from preferential encapsidation of defined species of molecules.

Comparison of the DNA of the newly isolated defective parvovirus (AAV 5) with the DNAs of the other four AAV serotypes by blot-hybridization experiments shows only a weak cross-reactivity (Fig. 3B). This is confined to the DNA bands representing complete genomes and, to a lesser extent, to the variant DNA of the largest size. On the other hand, rather strong cross-hybridization is seen between the other four AAV serotypes, and this reaction extends to smaller variant DNAs as well (Fig. 3C). These findings not only show that the isolated parvovirus is indeed different from AAV 1-AAV 4, but also demonstrate that AAV 5 is more distantly related to the four adeno-associated viruses than these are among each other. Since the variant DNAs show increasing deletions for internal regions as their size decreases but retain the genome termini (de la Maza and Carter, 1980), the homologies in the DNA of AAV 5 and AAV 1-AAV 4 are obviously located within the internal region. This assumption is supported by the finding that radiolabeled AAV 3 DNA shows no cross-hybridization with the terminal B-fragment of the *Xho*I cleavage and the terminal C-fragment of the cleavage with the enzyme *Bam*HI (Figs. 5, 7).

The concentration of unit length, linear duplex DNA in individual preparations usually is rather small (Fig. 2), and therefore a sensitive method was needed to determine the fragments obtained by digesting the double-stranded DNA with restriction enzymes. Since it was possible to transfer the DNA from polyacrylamide

gels to nitrocellulose filters (with experimental problems occurring only at low polyacrylamide concentrations) and to visualize it by filter hybridization, fragments down to 100 bp could be detected (Fig. 6). With this, no terminal fragments of the AAV 5 DNA smaller than the *Xho*I B-fragment (760 bp) and the *Eco*RI C-fragment (470 bp) were found for the enzymes tested, and mapping of the terminal region of the AAV 5 DNA remains to be done.

#### ACKNOWLEDGMENT

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 31: Tumorentstehung und -entwicklung).

#### REFERENCES

- ATCHISON, R. W., CASTO, B. C., and HAMMON, W. McD. (1965). Adenovirus-associated defective virus particles. *Science* 149, 754-756.
- BERNS, K. I., and ADLER, S. (1972). Separation of two types of adeno-associated virus particles containing complementary polynucleotide chains. *J. Virol.* 9, 394-396.
- BERNS, K. I., and ROSE, J. A. (1970). Evidence for a single-stranded adenovirus-associated virus genome: Isolation and separation of complementary single strands. *J. Virol.* 5, 693-699.
- BERNS, K. I., KORT, J., FIFE, K. H., GROGAN, E. W., and SPEAR, I. (1975). Study of fine structure of AAV-DNA with bacterial restriction endonucleases. *J. Virol.* 16, 712-719.
- BLACKLOW, N. R., CUKOR, G., KIBRICK, S., and QUINMAN, G. (1978). Interactions of adeno-associated viruses with cells transformed by herpes simplex virus. In "Replication of Mammalian Parvoviruses" (D. C. Ward and P. Tattersall, eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- BULLER, R. M. L., JANIK, J. E., SEBRING, E. D., and ROSE, J. A. (1981). Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. *J. Virol.* 40, 241-247.
- BÜDEMANN, H., and MÜLLER, W. (1978). Base specific fractionating of double stranded DNA: Affinity chromatography on a novel type of adsorbant. *Nucl. Acids Res.* 5, 1059-1074.
- CASTO, B. C., and GOODHEART, C. R. (1972). Inhibition of adenovirus transformation in vitro by AAV 1. *Proc. Soc. Exp. Biol. Med.* 140, 72-78.
- CHEUNG, A. K. M., HOGGAN, M. D., HAUSWIRTH, W. W., and BERNS, K. I. (1980). Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. *J. Virol.* 33, 739-748.

- DENHARDT, D. T. (1966). A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23, 641-646.
- FIFE, K. H., BERNIS, K. I., and MURRAY, K. (1977). Structure and nucleotide sequence of the terminal repetition in adeno-associated virus DNA. *Virology* 78, 475-487.
- GEORG-FRIES, B., BIEDERLACK, S., WOLF, J., and ZUR HAUSEN, H. (1984). Analysis of proteins, helper dependence and seroepidemiology of a new human parvovirus. *Virology* 134, 64-71.
- GERRY, H. W., KELLY JR., T. J., and BERNIS, K. I. (1973). Arrangement of nucleotide sequences in adeno-associated virus DNA. *J. Mol. Biol.* 79, 207-225.
- GRAHAM, F. L., SMILEY, J., RUSSEL, W. C., and NAIRN, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36, 59-72.
- HENLE, G., and HENLE, W. (1966). Immunofluorescence in cells derived from Burkitt's lymphoma. *J. Bacteriol.* 91, 1248-1256.
- HOGGAN, M. D. (1971). Small DNA viruses. In "Comparative virology" (K. Maramorosch and E. Kurstak, eds.), p. 43-74. Academic Press, New York.
- HOGGAN, M. D., BLACKLOW, N. R., and ROWE, W. P. (1966). Studies of small DNA viruses found in various adenovirus preparations: Physical, biological, and immunological characteristics. *Proc. Natl. Acad. Sci. USA* 55, 1467-1471.
- JEPPESEN, P. G. N. (1980). Separation and isolation of DNA fragments using linear polyacrylamide gradient gel electrophoresis. In "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 65, pp. 305-319. Academic Press, New York.
- KELLY, R. G., COZZARELLI, N., DEUTSCHER, M. P., LEHMANN, I. R., and KORBERG, A. (1970). Enzymatic synthesis of deoxyribonucleic acid. *J. Biol. Chem.* 245, 39-45.
- KIRSCHSTEIN, R. L., SMITH, K. O., and PETER, E. A. (1968). Inhibition of adenovirus 12 oncogenicity by adeno-associated virus. *Proc. Soc. Exp. Biol. Med.* 128, 670-673.
- KOCZOT, F. J., CARTER, B. J., GARON, C. F., and ROSE, J. A. (1973). Self-complementary of terminal sequences within plus or minus strands of adenovirus-associated virus DNA. *Proc. Natl. Acad. Sci. USA* 70, 215-219.
- KOLLER, B., DELIUS, H., BÜNEMANN, H., and MÜLLER, W. (1978). The isolation of DNA from agarose gels by electrophoretic elution onto malachite green-polyacrylamide columns. *Gene* 4, 227-239.
- LAUGHLIN, C. A., MYERS, M. W., RISIN, D. L., and CARTER, B. J. (1979). Defective interfering particles of the human parvovirus adeno-associated virus. *Virology* 94, 162-174.
- LUSBY, E., FIFE, K. H., and BERNIS, K. I. (1980). Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. *J. Virol.* 34, 402-409.
- LUSBY, E., BOHENZKY, R., and BERNIS, K. I. (1981). Inverted terminal repetition in adeno-associated virus DNA: Independence of the orientation at either end of the genome. *J. Virol.* 37, 1083-1086.
- MANIATIS, T., FRITSCH, E. F., and SAMBROOK, J. (1982). "Molecular Cloning, A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- MAYOR, H. D., TORIKAI, K., MELNICK, J. L., and MANDEL, M. (1969). Plus and minus single-stranded DNA separately encapsidated in adeno-associated satellite virions. *Science* 166, 1280-1282.
- MAYOR, H. D., HOULDTCH, G. S., and MUMFORD, D. M. (1973). Influence of adeno-associated satellite virus on adeno-virus induced tumors in hamsters. *Nature New Biol* 241, 44-46.
- DE LA MAZA, L. M., and CARTER, B. J. (1980). Molecular structure of adeno-associated virus variant DNA. *J. Biol. Chem.* 255, 3194-3203.
- DE LA MAZA, L. M., and CARTER, B. J. (1981). Inhibition of adenovirus oncogenicity in hamsters by adeno-associated virus DNA. *J. Natl. Cancer Inst.* 67, 1323-1326.
- MOUSSET, S., and ROMMELAERE, J. (1982). Minute virus of mice inhibits cell transformation by simian virus 40. *Nature (London)* 300, 537-539.
- MUSTER, C. J., LEE, Y. S., NEWBOLD, J. E., and LEIS, J. (1980). Physical mapping of adeno-associated virus serotype 4 DNA. *J. Virol.* 35, 653-661.
- OSTROVE, J. M., DUCKWORTH, D. H., and BERNIS, K. I. (1981). Inhibition of adenovirus-transformed cell oncogenicity by adeno-associated virus. *Virology* 113, 521-533.
- PARKER, R. C., and SEED, B. (1980). Two-dimensional agarose gel electrophoresis, "Sea-plaque" agarose dimension. In "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 65, pp. 358-363. Academic Press, New York.
- PARKS, W. P., MELNICK, J. L., RONGEY, R., and MAYOR, H. D. (1967). Physical assay and growth cycle studies of a defective adeno-satellite virus. *J. Virol.* 1, 171-180.
- RIGBY, P. W. J., DIECKMANN, M., RHODES, C., and BERG, P. (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* 113, 237-251.
- ROSE, J. A., BERNIS, K. I., HOGGAN, M. D., and KOCZOT, F. J. (1969). Evidence for a single-stranded adenovirus-associated virus genome: Formation of a DNA density hybrid on release of viral DNA. *Proc. Natl. Acad. Sci. USA* 64, 863-869.
- SHARP, P. A., SUGDEN, B., and SAMBROOK, J. (1973). Detection of two restriction endonuclease activities in Haemophilus parainflunzae using analytical agarose. *Biochemistry* 12, 3055-3063.
- SMITH, G. E., and SUMMERS, M. D. (1980). The bidirectional transcription of the adeno-associated virus genome. *J. Virol.* 34, 109-123.
- SOUTHERN, E. (1975). Detection of specific sequences among DNA fragments separated on polyacrylamide gels. *J. Mol. Biol.* 100, 39-56.
- TOOLAN, H. W. (1975). The isolation and characterization of H-1 virus of adenovirus. *J. Virol.* 52, 109-123.
- TOOLAN, H. W. (1975). The isolation and characterization of H-1 virus of adenovirus. *J. Virol.* 52, 109-123.

- Vol. 34, 402-403.
1. I. (1981). Association of H-1 virus with DNA at electron microscope. *Anal. Biochem.* 109, 103-108.
2. K. J. (1982). "H-1 virus." Cold Spring Harbor, New York.
3. J. and MANDEL. (1982). H-1 virus associated satellite DNA. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
4. MUMFORD, J. (1982). H-1 virus associated satellite DNA in hamsters. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
5. J. Molecular Biology of DNA. (1982). H-1 virus associated satellite DNA. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
6. Inhibition of H-1 virus by adenovirus 12. *Proc. Natl. Acad. Sci. USA* 67, 1323-1324.
7. Minute virus of man. *Proc. Natl. Acad. Sci. USA* 67, 1323-1324.
8. J. and LEIS. (1982). H-1 virus associated with adenovirus 12. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
9. BERNIS, K. I. (1982). H-1 virus associated with adenovirus 12. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
10. Dimensional analysis of H-1 virus. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
11. J. and MAYOR. (1982). H-1 virus associated with adenovirus 12. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
12. J. and BERG. (1982). H-1 virus associated with adenovirus 12. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
13. J. and KOCZOT. (1982). H-1 virus associated with adenovirus 12. *Proc. Natl. Acad. Sci. USA* 79, 103-108.
14. K. J. (1973). H-1 virus associated with adenovirus 12. *Proc. Natl. Acad. Sci. USA* 70, 103-108.
15. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyl-oxymethyl-paper. *Anal. Biochem.* 109, 123-129.
16. SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98, 503-517.
17. TOOLAN, H. W. (1967). Lack of oncogenic effect of the H-1 virus for hamsters. *Nature (London)* 214, 1036.
18. TOOLAN, H. W., and LEDINKO, N. (1968). Inhibition by H-1 virus of the incidence of tumors produced by adenovirus 12 in hamsters. *Virology* 35, 475-478.
19. TOOLAN, H. W., RHODE, III, S. L., and GIERTHY, J. F. (1982). Inhibition of 7,12 dimethylbenz(a)anthracene-induced tumors in syrian hamsters by prior infection with H-1 parvovirus. *Cancer Res.* 42, 2552-2555.
20. WARD, D. C., and TATTERSALL, P. (eds.) (1978). "Replication of mammalian parvoviruses," pp. 523-526. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
21. WEISLANDER, L. (1979). A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal. Biochem.* 98, 305-309.